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ISOLATION AND CHARACTERIZATION OF AN ANAEROBIC NITROGEN FIXING XYLANOLYTIC BACTERIAL CONSORTIUM FROM VERMICOMPOST AND ITS APPLICATION IN IMPROVING THE PROCESS OF VERMICOMPOSTING

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ABSTRACT

A stable anaerobic nitrogen fixing xylanolytic bacterial consortium was isolated from 45th day pressmud: bagasse: trash (8:1:1) vermicompost. It was designated as NBX-1 and consisted of Gram positive rods and cocci. The members of the consortium were inseparable from each other and therefore represent a stable symbiotic entity. It showed xylanase (8.4 µmols/ml/min) and nitrogenase (5024 nmols/hr/mg cell protein) activities. The individual functions of the partners could not be determined due to the inability to purify them. Combined inoculation of NBX-1 & earthworms (Eudrilus eugeniea) decreased the C/N ratio almost two weeks ahead of natural composting and thus improved the composting rate. 13C CPMAS NMR data highlights the distinct changes in the occurrence of various functional groups like olefinic, methylene and carbonyl groups in the compost on earthworm & NBX-1 inoculation.

INTRODUCTION

Several of the anaerobic environments where cellulose/hemicellulose is degraded lack combined nitrogen (Monserrate et al., 2001). Therefore obligate anaerobic bacteria and fungi which could degrade biopolymers like cellulose/hemicellulose and use this energy to fix atmospheric nitrogen, either independently or symbiotically, are of considerable significance. Such organisms can be applied as bio-fertilizers as they can supplement the

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lacking nitrogen (an essential nutrient) and augment the degradation of complex cellulose/hemicellulose molecules into easily absorbable forms for plant growth.

Free living diazotrophs in soils are able to use cellulose/lignocellulose decomposition products as energy sources for N_2 fixation (Halsall and Goodchild, 1986). Many obligately anaerobic cellulolytic species e.g., *Clostridium papyrosolvens* (Madden *et al.,* 1982), *Clostridium papyrosolvens* C7 (Leschine and Canale-Parola, 1983) strain JW-2 (Warshaw *et al.,* 1985) are reported to exhibit ammonium-repressible nitrogenase action. *Clostridium hungatei sp. nov.* (Leschine *et al.,* 1988; Monserrate *et al.,* 2001), an obligate anaerobe could be grown on xylan in the absence of a combined nitrogen source. But, except in the case of *Clostridium hungatei*, a detailed study on the enzyme activities of such organisms have not been reported.

Earthworms feed on soil and wood which could lack combined nitrogen. Therefore occurrence of nitrogen fixers in earthworm gut for the maintenance of C/N ratio cannot be ruled out. Many nitrogen fixing bacteria have been isolated from the gut of earthworms (Karsten & Drake, 1994).

The gut of earthworm is reported to be a mobile anoxic microzone containing high concentrations of organic substrates. These conditions stimulate a certain population of bacteria in the ingested matter which include denitrifying and fermentative bacteria (Horn *et al.,* 2003; Drake and Horn, 2007). Thus, earthworms exert an impact on terrestrial nitrogen cycle via the microbes which are transiently hosted in their gut.

The earthworm gut has been described as a "mutualistic digestive system", in which the exoenzymes released by the ingested microbes enhance the degradation of complex organic matter (Brown and Doube, 2004). Digestive enzymes (cellulases, chitinases, lipases etc) are secreted in to the intestine both by the worm and the ingested microorganisms.

Another habitat which provides a similar anoxic condition for the growth of such anaerobes is the vermicompost itself. Vermicomposting though widely considered an aerobic process harbours many anaerobic pockets, which serve as a microhabitat for obligate anaerobes (News Letter, ENVIS Centre for Environmental Biotechnology, 2006). Such anaerobic xylan degrading nitrogen fixers can be enriched and reinoculated into vermicompost and applied in fields. As these organisms are more adapted to the compost environment, they will have a better chance of survival and could accelerate the composting process. Compost prepared by the combined action of worms and microbes can also be then commercialized as value added vermicompost.

MATERIALS AND METHODS

2.1 Chemicals

Birch wood xylan was obtained from Sigma, USA and other chemicals were of analytical grade and the best quality available commercially.

2.2 Enrichment of Nitrogen Fixing Xylanolytic/Cellulolytic Anaerobes

a) Source of Inoculum: Vermicompost (prepared by mixing sugar industry residues like pressmud, bagasse and trash in the ratio 8:1:1). *Eudrilus eugeniea* species of earthworms were used. Inoculum used for enrichment was 10 mg per 50 ml broth.

(i) **Preparation of Vermicompost**: Pressmud was obtained from EID Parry, Nellikuppam, Tamil Nadu, India. Sugar cane trash and bagasse were obtained from local sites. Earthworms were procured from Department of Zoology, Annamalai University, Tamil Nadu, India and CPCRI (Central Plantation Crops Research Institute), Kasargode, Kerala, India.

Fresh pressmud obtained from the sugar industry was air dried before setting up the experiments to reduce the heat and odour. Bagasse and trash were cut into small pieces. Pressmud bagasse trash in the ratio of 8:1:1 (w/w) were mixed well and moistened with water. The mixture was then left for precomposting for 3-4 days in clay pots. The precomposting is necessary to decrease the initial heat generated as a result of rapid microbial activity. After this precomposting stage earthworms (*Eudrilus eugeniea*) were inoculated (15 g/kg) into the pots. Composting was carried out for 50 days.

(ii) **Sample Collection**: Vermicompost samples: Samples were collected from each pot after mixing the whole ptb mixture from top to bottom.

b) Growth Medium Preparation: Anaerobic medium preparation and cell cultivation were done by the modified Hungate technique (Hungate, 1969) using serum vials (Miller and Wolin, 1974). The nitrogen-free mineral base medium consisted of (in g/L): KH_2PO_4: 0.45; K_2HPO_4: 0.45; NaCl: 0.9; $MgSO_4.7H_2O$: 0.18; $CaCl_2.2H_2O$: 0.12; $FeSO_4.7H_2O$: 0.1; $MnSO_4$: 0.5; $CoCl_2$: 0.1; $ZnSO_4$: 0.1, $CuSO_4$: 0.01; $AlK(SO_4)_2$: 0.01; H_3BO_3: 0.01; Na_2MoO_4: 0.01, resazurin: 0.001 (Murthy and Chandra, 1989). Nitrogen gas was deoxygenated by passing through heated copper column kept at 350 ˚C and hydrogen gas was passed simultaneously for the reduction of copper. The medium was boiled for 5 minutes and then cooled under deoxygenated nitrogen gas. After cooling, 0.5 g of solid cysteine hydrochloride was added and required volumes were removed into serum vials using 5-50 ml syringes. The vials were sealed by crimping and autoclaved for 15 minutes at 121 °C. After cooling the pH was checked using pH paper. If the pH was below 7.0 then 150 µl of filter sterilized stock solution of $NaHCO_3$ (10 %) was added to the vials using hypodermic syringes to give a final pH of 7.0 ± 0.2.

c) Carbon Source: Birchwood xylan 0.5 % was added directly to the medium before autoclaving. Filter paper cellulose (Whatman No. 1) was cut into strips of size 1x5 cm and added to the medium in the vials before autoclaving.

d) Growth Conditions: The culture broth in sealed anaerobic vials was incubated at 30 °C without shaking.

2.3 Characterization of the Enriched Stable Microbial Consortia

(a) Microscopic studies: The enriched microbial consortium was examined under a light microscope after Gram staining. Scanning electron microscopy was also employed to study the morphology of the cultures. After pelleting and washing, the cells were prepared for SEM (Albrecht *et al.,* 1976).

(b) Colony Characteristics and Biochemical Properties: The morphological and physiological properties of the isolated consortium was studied by adopting the methods recommended in the Manual of Methods for General Bacteriology (Gerhardt *et al*., 1981). Biochemical tests, like utilization of various sugars was studied by adding 0.5 % (w/v) of the substrates to the mineral base medium.

(c) 16S rRNA Gene Isolation: Fresh biomass (about 200 mg) was blot dried and ground with Carlson lysis buffer (Carlson *et al.,* 1991) using mortar and pestle for DNA extraction.

(d) Preparation of Carlson Lysis Buffer (100 ml): 100 mM Tris, pH 9.5 (1.21g Tris base in 70 ml ddH_2O, pH to 9.5 with HCl), 20 mM EDTA (0.76 g), 1.4 M NaCl (8.18 g), 2 % CTAB (2 g) [CTAB = Hexadecyltrimethylammonium bromide, Sigma H-5882], 1 % PEG 8000 or 6000 (1 g) [PEG = polyethylene glycol] were mixed and stirred until dissolved (sometimes overnight). Made up to 100 ml and added 2 µl of β-mercaptoethanol per 1 ml of buffer just before use.

Specific 16S rRNA gene Universal Eubacterial primers were used for PCR amplification after DNA extraction (Weisburg *et al.,* 1991).

Forward FD1 5′ GAGTTTGATCCTGGCTCAG

Reverse rP2 5′ ACGGCTACCTTGTTACGAC

The PCR program used included one cycle of denaturation at 94 °C for 2 minutes, 30 cycles of 94 °C (45 seconds), 60 °C (30 seconds) and 72 °C (1.5 minutes), followed by a final elongation step of 10 minutes at 72 °C. The amplified product was run on 0.8 % agarose gel and compared with a 500 bp DNA ladder for checking the presence of 16S rRNA gene in the sample.

(e) Measurement of Growth: This was performed by estimating the total cell protein by Lowry method (Lowry *et al*., 1951) using Bovine Serum Albumin as working standard.

(f) Estimation of Xylan Utilization: This was done by phenol sulphuric acid method (Dubois *et al*., 1956.

(g) Estimation of Total Nitrogen in the Culture Supernatant and Pellet: Total nitrogen was determined by micro-kjeldahl method by digesting the sample (0.2 ml) with 2 ml concentrated sulphuric acid on a mantle. After cooling, few drops of H_2O_2 were added and the digestion was continued till the solution became clear. 8 ml of 40% NaOH was added to the digest on cooling and distilled into a conical flask containing 10 ml of 4 % boric acid and 2-3 drops of bromocresol green indicator. Distillation set was maintained airtight. After distillation the contents in the conical flask was titrated against 0.01 N H_2SO_4 till the original green colour was obtained. Distilled water was used as blank (Pellett and Young, 1980).

(h) Analysis of fermentation end products: The general procedure of Holdeman *et al.,* (1977) was used for the analysis of volatile end products using gas chromatograph fitted with flame ionization detector (FID). Cell free culture filtrate was obtained by centrifuging broth at 12,000x g for one hour at 4 °C. Working standard solutions (1-5 mM) of ethanol, butanol, isopropanol, acetone, 2-3 butane diol, acetic, propionic and butyric acids were prepared. To 2 ml of the culture broth or working standard solution, 0.4 ml of 50 % sulfuric acid and 1 ml of 0.9 % NaCl were added and extracted with 1 ml of CCl_4. Chromosorb 101 column was used for analysis with N_2 as carrier gas at a flow rate of 35 ml min^{-1}, H_2 at 25 ml min^{-1} and air at 200 ml min^{-1} with injector, detector and column temperatures set at 210, 210 and 190 °C respectively.

(i) Enzyme Assays:

(i) **Xylanase (EC 3.2.1.8):** Xylanase activity was determined by measuring released reducing sugars using the DNS (dinitrosalicylic acid) method (Miller, 1959).

(ii) **Nitrogenase (EC 1.18.6.1):** The acetylene reduction test of Postgate *et al*. (1972) was done using gas chromatography (AIMIL NUCON 5764 GC; FID, Poropak N column). Three ml of the culture broth was retrieved from the anaerobic culture broth and transferred into 25 ml serum vials and closed with butyl rubber stoppers and crimped with aluminium seals. Five ml commercially available pure acetylene gas (Indo Gas Agencies, Chennai, India) was injected into the vial after removing the same amount of air from it. Gas sample (0.5/1.0 ml) was retrieved from the bottles after specific time intervals using a gas tight syringe and injected into the GC column. The oven, injection port, detector temperatures were 60, 90 and 110 °C respectively. Ethylene gas was used as standard. It was prepared and the samples withdrawn in the laboratory.

2.4 Application of the Enriched Anaerobic Nitrogen Fixing Xylanolytic Consortium in Improving the Process of Vermicomposting Experimental Set-up:

Sugar industry wastes pressmud, trash and bagasse were used for compost preparation as described earlier. Three clay pots each containing 2 kg of the pressmud : trash: bagasse mixture (8:1:1) for natural composting (control pot) and composting with NBX-1 inoculation were set up. Six pots were set up with NBX-1 plus earthworms (15 g/kg).

a) Inoculation of the Enriched Consortium into the Vermicompost Pots: NBX-1 (50 ml broth after 8 days of growth, approx. 85mg total cell protein) was added to the pots every 10 days and mixed well. NBX-1 was also added to the ptb mixture compost set up without the worms. The process was monitored for 50 days.

b) Sample Collection: Samples were taken after mixing the contents of the pot from top to bottom. Approximately 200 gm of sample (mixture of sub samples) were collected from each pot every 10 days. The collected vermicompost samples were oven dried at 100 °C, then ground in blender and sieved with a 2 mm diameter sieve prior to analysis.

c) Analysis of Total Carbon: Samples which were smaller than 2 mm in diameter were used for analysis. Total carbon was estimated by the wet oxidation method of Walkley and Black (1934).

d) Estimation of Total Nitrogen: Total nitrogen was determined by macro-Kjeldahl method (Jackson, 1958).

e) ^{13}CP/MAS NMR Spectroscopy: The ^{13}C NMR spectra were obtained with CP/MAS using a Bruker Avance 400 MHz NMR spectrometer. The spectrometer was operated at a ^{1}H frequency of 400 MHz and a ^{13}C frequency of 100 MHz with contact time of 1ms, recycle delay of 5s, sweep depth of 31250 Hz, line broadening of 100 Hz and spinning speed of 8 KHz. The chemical shift was calibrated to tetramethyl silane (=0 ppm). For interpretation of the spectra the following ranges and preliminary assignations were considered: region I (0-50 ppm) aliphatic carbon; region II (50-110 ppm) O-alkyl carbon; region III (110-160 ppm) unsaturated and aromatic carbon; region IV (160-190) carboxyl carbon.

RESULTS AND DISCUSSION

3.1 Enrichment of Nitrogen Fixing Xylanolytic/Cellulolytic Anaerobes

No microbial growth was obtained when filter paper cellulose was used as the sole carbon source for growth. A stable anaerobic nitrogen fixing xylanolytic bacterial consortium which was designated NBX-1 could be enriched from 45th day vermicompost.

3.2 Characterization of NBX-1

a) Cell and Colony Morphology: NBX-1 is a bacterial consortium consisting of Gram positive rods and cocci. There was delayed colony formation on agar slants When the colonies were picked up and inoculated separately they failed to grow, indicating that the consortium NBX-1 is inseparable and therefore represents a single stable entity.

A member of the bacterial consortia NBX-1 was concluded to be belonging to the genus *Clostridia* by microscopic examination. Symbiosis can be stated as a potential reason for the delayed colony formation of NBX-1 on agar slants and roll tubes.

b) Growth Characteristics: The consortium NBX-1 was obligately anaerobic. No growth could be obtained under aerobic or microaerophilic conditions (tested by growing the cultures in tubes under aerobic conditions with a layer of sterile paraffin oil on top to provide microaerophilic conditions). NBX-1 like *Clostridium hungatei sp. nov.* (Leschine *et al.,* 1988; Monserrate *et al.,* 2001) showed growth on various carbon sources like glucose, xylose, birch wood/oatspelt xylan, CMC, mannitol, pectin, maltose etc (**Table 1**). The total cell protein content reached about 1.6 mg/ml by the 8th day of growth (**Fig. 1**). An inverse relation was observed between the residual substrate in the media and increase in cell protein (**Fig. 1**). Acetic acid was the predominant end product of metabolism of NBX-1 as confirmed by gas chromatography analysis. Ethanol, butyric acid and propionic acid were also formed in trace amounts. Analysis was done at the end of 8th day of growth. pH of the growth medium decreased from 7.0 to 3.5 by the 10th day of growth. The decrease in media pH with growth of NBX-1 can be linked to the production of acetic acid as a major end product of metabolism.

c) Xylan Utilization and Xylanase Activity: NBX-1 showed a maximum xylanase activity of 8.4 µmols/ml/min under optimized conditions by the 6th day of growth (**Fig. 2**). The optimum conditions for xylanase activity were pH: 5.5, enzyme volume: 0.2 ml (0.03 mg protein), incubation time: 20 minutes and temperature: 50 °C. The increase in enzyme activity was parallel to increase in extracellular protein (**Fig. 2**).

NBX-1 showed good xylanase activity. The cellulosomes and xylanosomes of anaerobic bacteria have been a topic of intensive research over the years. The cellulosome complex of *Clostridium pasturianum* is the best studied till date. NBX-1 is similar to the pure culture *Clostridium hungatei* as both can utilize xylan as an energy source for nitrogen fixation. **Table 3** gives a comparison of the nitrogenase and xylanase activities of *Clostridium hungatei* & NBX-1. The xylanase activity of *Clostridium hungatei* is 14 µmols/ml/min while that of NBX-1 is 8.4 µmols/ml/min.

d) Nitrogen Fixation and Nitrogenase Activity: There was a marked increase in the total nitrogen content in the supernatent and pellet during the growth of NBX-1 (**Fig. 3 a**). Nitrogenase activity peaked around the 8th day of growth in NBX-1 (**Fig. 3 b**).

Line and Loutit (1973) faced difficulties in separating out similar anaerobic nitrogen fixing consortia and have briefly reported N_2-fixing *Clostridia* in mixed cultures derived from soils. Later Minamisawa *et al.,* (2004) have reported that a major process that supports the growth and N_2-fixing activity of the anaerobic ***Clostridium*** is oxygen elimination by the accompanying nondiazotrophic bacteria ***Enterobacter cloacae*** and ***Bacillus megaterium***, which enables the consortium to fix nitrogen in a seemingly aerobic environment. Anaerobic nitrogen fixing consortia are widespread in wild rice species and pioneer plants, which are able to grow in unfavourable locations. Minamisawa's (2004) studies indicate that *Clostridia* are naturally occurring endophytes in gramineous plants like sugarcane and that clostridial nitrogen fixation arises in association with nondiazotrophic endophytes. This fact is reaffirmed by the present study.

A major feature of anaerobic nitrogen fixing consortia is that nitrogen fixation by the anaerobic *Clostridia* is supported by the elimination of oxygen by the accompanying bacteria in the culture. In a few anaerobic nitrogen fixing consortia, non-diazotrophic bacteria specifically induced nitrogen fixation of the *Clostridia* in culture (Minamisawa *et al.,* 2004). During their examination of the N_2-fixing activity profiles of the clostridial isolates with accompanying bacteria, Minamisawa *et al.,* (2004) noticed that three clostridial isolates (Kas107-2, OkiF101, and OkiN108) expressed no N_2- fixing activity in single cultures, even in an anoxic environment. However, they showed N_2-fixing activities in coculture with some isolates of accompanying bacteria from identical plant tissues. All of the clostridial isolates they tested displayed the accompanying bacterium-dependent N_2 fixation. This suggests the presence of exchangeable, specific anaerobic nitrogen fixing consortia relationships for the expression of N_2 fixation. We presume the occurrence of a similar phenomenon in explaining the high levels of nitrogenase activity displayed by NBX-1. Further examinations were done to ascertain as to whether the accompanying bacteria produced specific metabolites that induced the N_2 fixation of the clostridial isolate. The addition of a specific culture filtrate of ***Bacillus*** sp. strain Kas107-3 caused the expression of N_2-fixing activity in ***Clostridium*** sp. strain Kas107-2, whereas a Kas107-4 filtrate did not. These data suggest that N_2 fixation in anaerobic nitrogen fixing consortia is dependent on at least two factors, the presence of unknown metabolites and low O_2 concentrations.

NBX-1 consortium was enriched from 45^{th} day vermicompost prepared by mixing sugar industry wastes like pressmud, trash and bagasse in the ratio 8:1:1. *Eudrilus* species earthworms were used (15 gm/Kg). It is interesting to note that similar consortia could not be enriched from the earthworm gut which is known to harbour an anoxic environment. There are various implications of anaerobic nitrogen fixing consortia in microbial ecology and nitrogen fixation. Their sensitivity to molecular oxygen generally restricts ***Clostridium*** sp. to anaerobic areas such as water, submerged soil, rumina, and intestines (Catto *et al.,* 1986; Hippe *et al.,* 1992). The plant-dwelling *Clostridia* probably sometimes proliferate in anoxic microzones produced by anaerobic nitrogen fixing consortia or plant respiration, while they survive in spore forms under higher O_2 concentrations. Interestingly, this type of anaerobic nitrogen fixing consortia reported by Minamisawa *et al.,* (2004) includes two clostridial isolates (OkiF101 and OkiN108) from sugarcane. Sugarcane has been intensively studied for biological N_2 fixation by bacterial endophytes (James, 2000; Boddy *et al.,* 1995; Sevilla *et al.,* 2001). The concept of anaerobic nitrogen fixing consortia enables us to recognize and isolate N_2-fixing *Clostridia* from plants.

The work of Minamisawa *et al.,* (2004) explains the apparent unculturability of N_2-fixing anaerobes by the conventional procedure of single-colony isolation as was observed in the present study too. Indeed, a survey work on the purification of N_2-fixing microbes from pasture grasses in Southeast Asia had failed due to the problem of the apparent unculturability of diazotrophs (Minamisawa *et al.,* 2004).

3.3 Application of the Enriched Anaerobic Nitrogen Fixing Xylanolytic Bacterial Consortium NBX-1 in Improving the Process of Vermicomposting

a) C/N Ratio: Application of NBX-1 in improving vermicomposting was studied by monitoring the effects of inoculation of NBX-1 into pressmud, bagasse, trash compost. The reduction in C/N ratio and thus the rate of composting was much prominent and faster in the pots inoculated with NBX-1 along with earthworms when compared to control and NBX-1 alone inoculated pots (**Fig. 6**). Prakash *et al.,* 2008 have reported that organic carbon and C/N ratio were lower in vermicompost and casts. The ideal C/N ratio for mature compost should be below 12 (Hsu and Lo, 1999). Total nitrogen, phosphorus, potassium, calcium, copper, iron, zinc etc are reported to be higher in vermicompost and cast when compared with worm unworked substrates (Prakash *et al.,* 2008).

Consolidated bioprocessing of agro residues into biofertilizers has gained much interest over the years. The method achieves substrate utilization and end product formation in a single process step (Lynd *et al*., 2002). Vermicomposting coupled with exogenous addition of microbial inoculants is reported to accelerate composting rate and enhance its manurial value (Kumar and Singh, 2000). Though exogenous addition of culture has been practiced with aerobes not much study has been done with anaerobes.

Maturing of compost involves several changes in the chemical content. It is an important factor which decides the successful application of compost in agriculture and its impact on the environment (Senesi and Brunetti, 1996; Chefetz *et al.,* 1996). The organic matter transformations during composting can be predicted from the C/N ratio and ^{13}C NMR analysis (Hsu and Lo, 1999).

b) ^{13}C CPMAS NMR Analysis: ^{13}C CPMAS NMR data reveals that changes in the abundance of functional groups like methylene, olefinic and carbonyl groups are occurring in the compost on the addition of NBX-1 and earthworms. The methylene, olefinic and carbonyl peaks have changed substantially in the spectra obtained from the three different experimental setups (**Fig. 7, Table 2**). **Table 2** shows the changes in the intensity of different organic functional groups in the ^{13}C CPMAS NMR spectra of control, NBX-1 and earthworm inoculated ptb compost after 50 days. The methylene group (25-45 ppm) is weak in control pots where as strong in the NBX-1 alone and NBX-1 + earthworm inoculated pots. Olefinic groups (100-107 ppm), which could be derived from pressmud are strong in control pots and NBX-1 alone inoculated ptb compost pots. Intensity of the carbonyl group (170-200 ppm) show a change similar to that of methylene group, i.e., weak in control pots where as strong in the NBX-1 alone and NBX-1 + earthworm inoculated pots**.**

Solid state ^{13}C CP/MAS NMR allows the direct chemical characterization of organic materials in compost. It is a non destructive and sensitive technique. Wilson *et al.,* (1981) acquired well resolved ^{13}C CP/MAS NMR spectra for soil organic matter. It provided useful data on the chemical structure of the sample and thus established the usefulness of the

technique as a tool for characterizing soil organic materials. In 2007 Sen and Chandra had successfully established the potential of the technique in characterizing vermicompost prepared from sugar industry wastes.

Solid state NMR has been proved to be useful to characterize the processes of microbial degradation in lignocellulosic materials (Baldock *et al.,* 1997). In the ^{13}C CPMAS NMR spectra alkyl groups are seen around 21 ppm (terminalmethyl) and 32 ppm (methylene in aliphatic rings and chains). The signal at 56 ppm is assigned to methoxyl in lignin (phenol methoxyl of coniferyl and sinapyl moieties) and in hemicellulose (glucuronic acid in xylan) (Veeken *et al.,* 2001). The region between 60-110 ppm gives peaks of proteins and polysaccharides. The signals around 76 and 83 ppm are due to C_2, C_3, C_5 and non-crystaline component of C_4 of cellulose. Signals derived from hemicellulose are contained within the cellulose peaks itself. The peaks around 115 and 120 ppm are due to H-aromatic C (Kogel-Knabner, 1997) and peaks around 140 ppm are due to aromatic carbon (Frund *et al.,* 1994). The peaks formed at 170-173 ppm are assigned to carboxylic, amide and ester groups (Preston *et al.,* 1990). In the present study, ^{13}C CPMAS NMR data shows that distinct changes in the functional groups like olefinic, methylene and carbonyl groups are occurring in the compost on earthworm and NBX-1 inoculation (**Table 2 Fig. 5**). But these changes in the intensity of the functional groups are not confirmative of the indication of any major structural changes occurring in the vermicompost as a result of NBX-1 or worm inoculation.

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APPENDIX

Table – 1: *Growth Characteristics of NBX-1 on Various Substrates Under Nitrogen Free Conditions*

Substrate	TCP (mg/ml)
Birchwood xylan	1.7
Oatspelt xylan	1.7
Xylose	1.8
Glucose	1.7
Carboxy methyl cellulose	0.6
Mannitol	0.5
Pectin	1.0
Maltose	0.7
Glycerol	0.3
Starch	0.6
Cellulose powder	0.4
Filter paper cellulose	No growth

TCP = Total Cell Protein Content on the 10th Day of Growth
Value Given is the Average of Four Values

Table – 2: *^{13}C CP/MAS NMR Analysis of Control, NBX-1 and Earthworm Inoculated Ptb Compost*

Ppm	Functional group	Control (ptb)	ptb + NBX-1	ptb + NBX-1 + EW
25-45	Methylene	weak	strong	Strong
100-107	Olefinic	strong	strong	Weak
170-200	Carbonyl	weak	strong	Strong

Table – 3: *Comparison of the Xylanase and Nitrogenase Activities of NBX-1 with Clostridium Hungatei*

Organism	Nitrogenase activity (nmols/hr/mg cell protein)	xylanase activity (µmols/ml/min)
Clostridium hungatei	700	14
NBX-1	5024	8.4

Monserrate, 2001; Leschine, 1998

Fig. 1: *Growth and Substrate Utilization of NBX-1 on 0.5 % Birchwood Xylan when Grown in the Absence of a Combined Nitrogen Source*

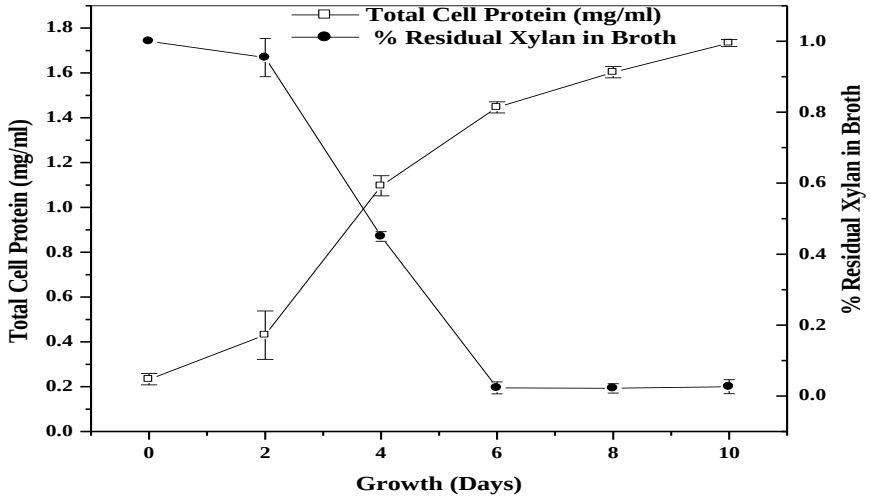


Fig. 2: *Xylanase Activity and Extracellular Protein Content of NBX-1.*

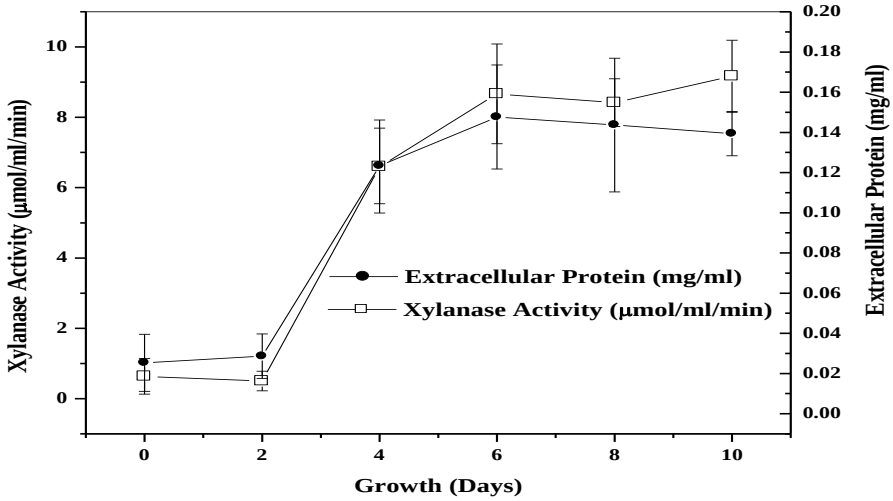
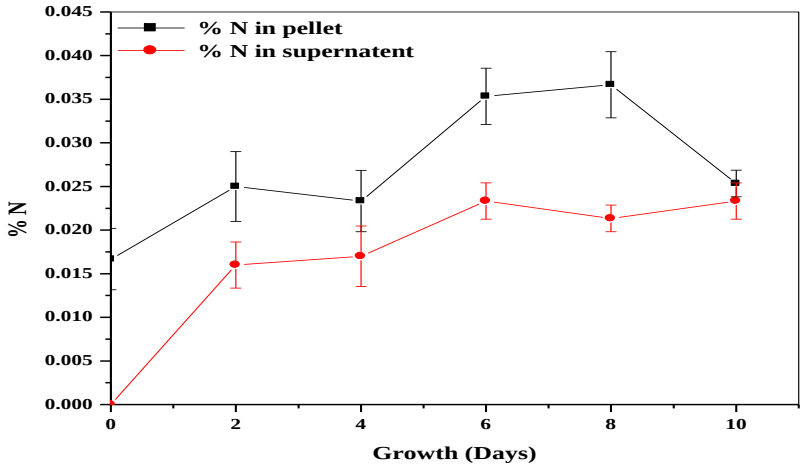


Fig.3 *(a) Increase in Total Nitrogen Content During Growth of NBX-1; (b) Time Course of Nitrogenase Activity of NBX-1*

(a)



(b)

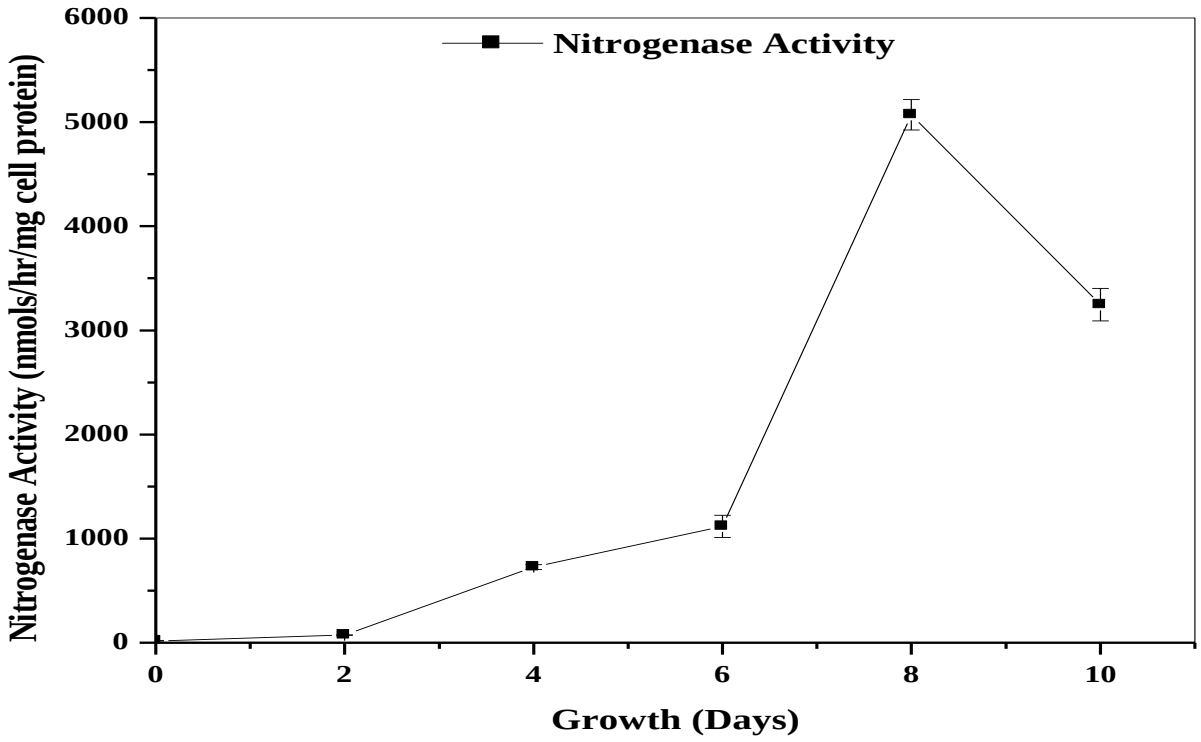


Fig4 *Graph Showing the Time Course of C/N in Control, NBX-1 and Earthworm Inoculated Compost Pots. Red, Green and Blue Lines Indicate the Time Taken for the Compost to Reach Maturity. Red, EW + NBX-1 Inoculated Pot; Green, NBX-1 Alone Inoculated Pot; Blue, Control Pot*

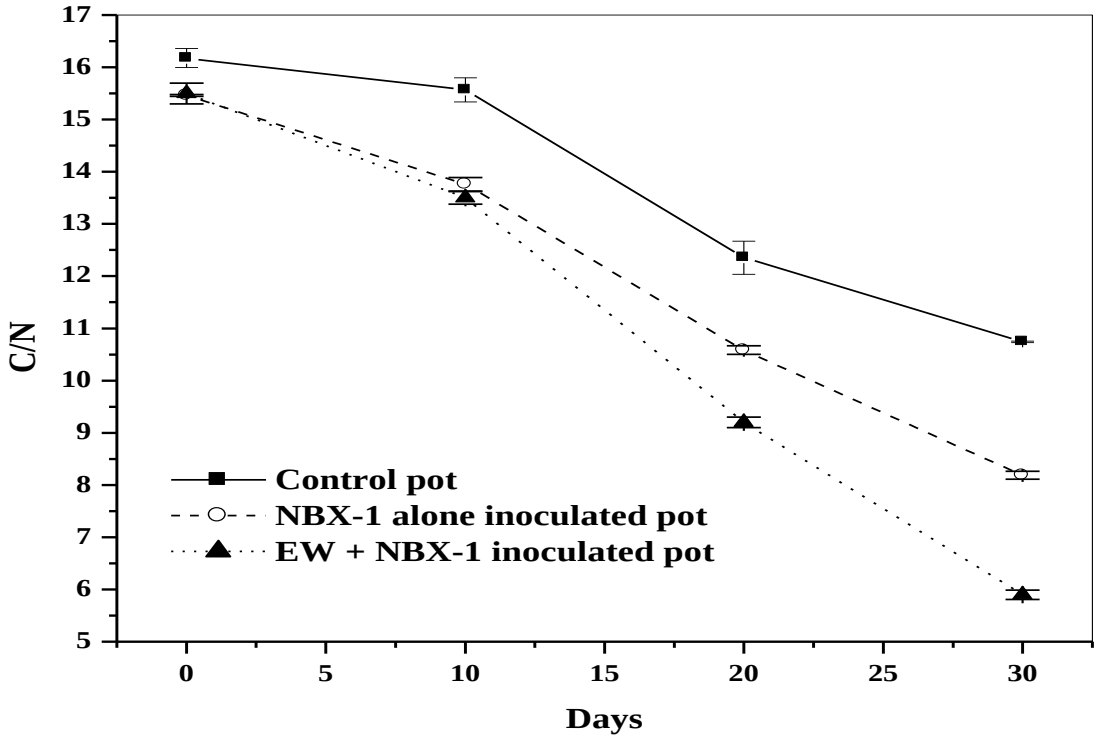
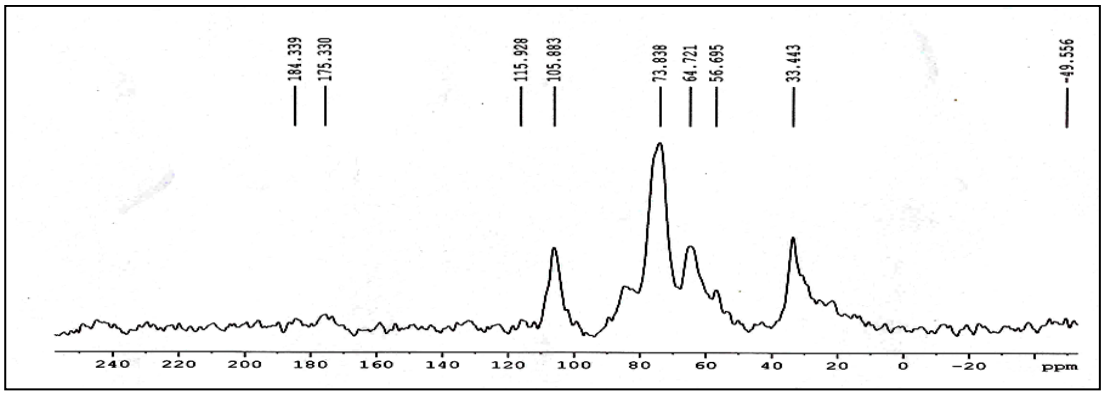
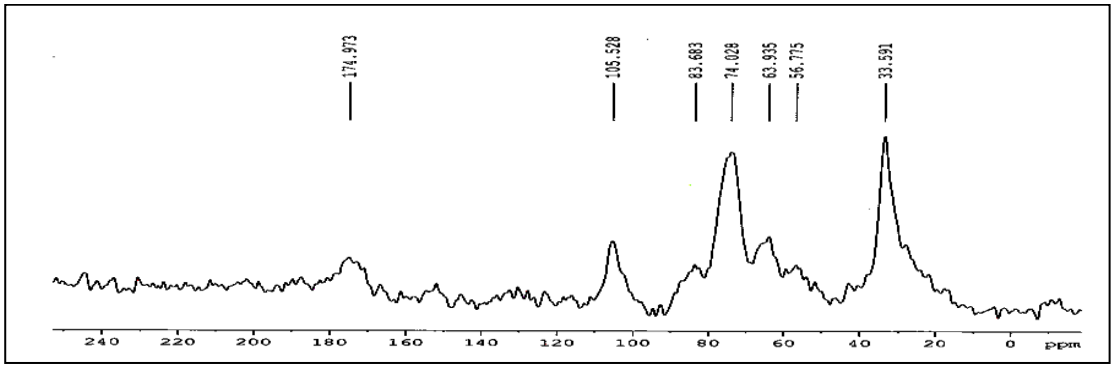


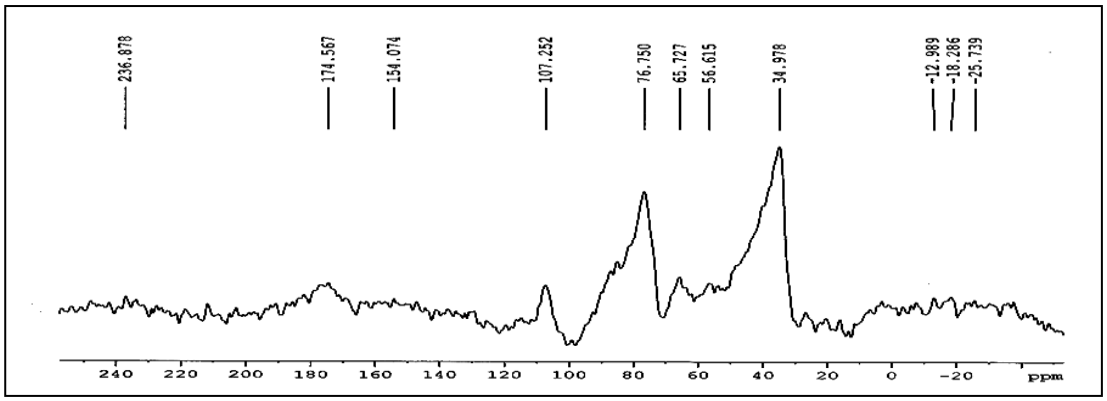
Fig.5 *^{13}C CP-MAS NMR Analysis Showing the Structural Changes Brought about as a Result of Inoculation of NBX-1 and/or Earthworm into Pressmud, Trash and Bagasse (PTB) Compost. A Comparison is Made with Natural Composting of the Substrates (Control). The Spectra Given here are of the 50th Day Samples.*



a) Control PTB Mixture (Natural Composting)



b) Ptb + NBX-1 Mixture Compost



c) Ptb + NBX-1 + Earthworm Mixture Compost